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Distinct and Overlapping Expression Patterns of Kinases during Mouse Embryogenesis

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Recent studies have implicated Eph-related receptor tyrosine kinases and their membrane-bound ligands in restricting or stimulating the movement of cells and axons. Members of these large families of receptors and ligands fall into two major binding specificity classes, in which the GPI-anchored subgroup of ligands can each bind to all members of a subgroup of receptors, whereas the transmembrane ligands interact with a distinct subgroup of receptors. Analysis of expression patterns is therefore important in order to understand which receptor–ligand interactions occur *in vivo*. We have cloned mouse orthologues of five members of the ligand family and analysed in detail their developmental expression, in comparison with each other, and with the receptor specificity class they can interact with. We find that B61, AL-1/RAGS, LERK4, and ELF-1, members of the GPI-anchored subgroup of ligands, have both distinct and overlapping aspects to their expression in early mesoderm, somites, and branchial arches; in complex, dynamic patterns in the limb; and in spatial domains and specific neurons in the CNS. Similarly, Elk-L is expressed in hindbrain segments, the roof plate, and floor plate, which overlaps with that of other transmembrane ligands, but has distinct expression in somites. The expression domains of ligands are complementary to those of the corresponding receptors in a number of tissues, including the midbrain, hindbrain, and differentiating limbs, consistent with potential roles in restricting cell movement. In addition, we find that there are some overlaps in expression of receptors and ligands, for example in somites and the early limb. Taken together with previous studies showing that Eph-related receptors also have distinct but overlapping expression patterns, these data indicate that each ligand may have stage- and tissue-specific interactions with an individual member or multiple members of the receptor family.

INTRODUCTION

Cell–cell signalling has a crucial role in regulating the proliferation, differentiation, and spatial patterning of tissues during embryogenesis, and thus important insights into these processes have come from the identification and functional analysis of extracellular signals and their receptors. An increasing number of signalling molecules have been implicated in development that are ligands for members of the receptor tyrosine kinase (RTK) superfamily. These receptors consist of an extracellular ligand-binding domain, a transmembrane region, and an intracellular tyrosine kinase domain, and their activation upon binding of ligand initiates an intracellular cascade leading to changes in cell behaviour and/or gene expression (reviewed by van der Geer *et al.*, 1994). Members of the RTK superfamily fall into distinct classes based upon structural motifs in the extracellular domain, and, within each of these, individual receptors (or alternatively spliced variants) have a distinct, but often overlapping binding specificity for ligands.

The best characterised extracellular signals implicated in vertebrate embryogenesis, such as FGFs and PDGFs, are secreted molecules that can diffuse several cell diameters or more from their site of synthesis. In contrast to such diffusible signals, recent work has indicated that ligands for Eph family RTKs may mediate signalling only between adjacent cells. Eph-related RTKs comprise the largest known family of receptors, with at least 13 members, re-

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lated to each other by the conservation of two fibronectin type III repeats and 20 cysteine residues in the extracellular domain (van der Geer *et al.*, 1994). Studies of expression during embryogenesis have revealed dynamic spatial and cell-type restricted patterns, suggesting that members of this family may cooperate in the development of a number of tissues (Nieto *et al.*, 1992; Becker *et al.*, 1994; Henkemeyer *et al.*, 1994; Patel *et al.*, 1996). For example, in the CNS at least 5 Eph family RTKs are segmentally expressed in the developing hindbrain (Nieto *et al.*, 1992; Becker *et al.*, 1994; Ganju *et al.*, 1994; Henkemeyer *et al.*, 1994; Ruiz and Robertson, 1994; Ellis *et al.*, 1995), and several members of this family have been shown to be expressed in developing neurons (Pasquale *et al.*, 1992; Henkemeyer *et al.*, 1994; Cheng *et al.*, 1995; Ohta *et al.*, 1996; Kilpatrick *et al.*, 1996). Taken together, these expression studies suggest a number of potential roles, including patterning of the mesoderm, central nervous system, and limb and the outgrowth of neurons.

Further clues regarding function have come from the identification of ligands that bind to and activate Eph family RTKs (Bartley *et al.*, 1994; Beckmann *et al.*, 1994; Cheng and Flanagan, 1994; Davis *et al.*, 1994; reviewed by Pandey *et al.*, 1995a). These ligands are a family of polypeptides that can be divided into two structural classes. Members of the first class have a consensus sequence for the attachment of glycosylphosphatidylinositol (GPI) that anchors the polypeptide in the plasma membrane, whereas members of the second class have a transmembrane domain and cytoplasmic tail. In contrast to membrane-bound ligand, soluble forms of these ligands are not active, possibly because clustering that is facilitated by membrane anchoring is required for receptor activation (Davis *et al.*, 1994). It therefore seems that these ligands mediate contact-dependent signalling between adjacent cells.

In addition to being unique in mediating signalling only between neighbouring cells, several lines of evidence suggest that Eph-related RTKs may regulate different cellular responses from other classes of RTKs. Although several Eph-related RTKs are highly expressed in some tumours (reviewed by Brambilla and Klein, 1995), activation of these receptors does not stimulate proliferation (Lhotak and Pawson, 1993; Brambilla *et al.*, 1995). One possibility is that these receptors instead regulate the migration or adhesion of cells, and this is consistent with the finding that PI3 kinase is a target of activated Eck receptor (Pandey *et al.*, 1994). Further support for this comes from initial functional analyses of specific Eph family receptors and their ligands. Use of a dominant negative strategy implicates the Eph family RTK, Sek-1, in the patterning of hindbrain segments and suggests that this receptor may either regulate the spatial identity or restrict the movement of cells between rhombomeres (Xu *et al.*, 1995). Studies of the ligand, B61, indicate a role in the migration of endothelial cells during angiogenesis mediated by activation of the Eck receptor (Pandey *et al.*, 1995a). Finally, independent lines of investigation have implicated the ligand, AL-1/RAGS, in fasciculation of axons of cortical neurons (Winslow *et al.*, 1995) and in the guidance of retinal axons to specific targets in the tectum (Drescher *et al.*, 1995).

Taken together, these studies provide the first indications of roles of Eph family RTKs and their ligands, and based on the widespread and complex expression of the receptors it would seem very likely that they have roles in many tissues. *In vitro* studies indicate that the Eph-related RTKs and their ligands can each be divided into two classes based on their binding specificities (Brambilla and Klein, 1996; Gale *et al.*, 1996a). Members of one class of receptors (Elk, Sek-3/Nuk, Hek2, and Htk) only interact with the transmembrane ligands (Elk-L/LERK2 and Htk-L/ELF-2), while the other class of receptors (Ehk-1, Ehk-2, Ehk-3, Hek, and Eck) only interact with the GPI-anchored ligands (ELF-1, Ehk1-L, LERK4, AL-1/RAGS, and B61); the only known exception to these binding specificity classes is the Sek-1 receptor that can interact with all GPI-anchored and some transmembrane ligands. The extracellular domain of receptors and ligands can be fused to the constant region of human IgG1 to create fusion proteins (receptor- or ligand-Fc) that can be used for the whole mount detection of the corresponding classes of ligands or receptors. Studies of 10.5 dpc and later stage mouse embryos have shown that GPI-anchored ligands detect similar patterns of receptor expression as each other, consistent with their similar binding specificity (Gale *et al.*, 1996a). Strikingly, these patterns are complementary to those of the ligands detected by the corresponding receptor-Fc fusions. Taken together, these data raise the possibility that an individual Eph-related RTK could interact with multiple ligands during development, and/or each ligand could interact with multiple receptors. It is therefore important to analyse the expression patterns of the ligands and to compare these to those of the receptors with which they can potentially interact. To obtain insight into this we have cloned mouse homologues of five members of the ligand family, analysed their expression patterns during early development, and compared these with the expression of the receptor and ligand binding specificity classes. We find complex and dynamic expression of ligands in many tissues, including the CNS, branchial arches, and the limb, and also that different ligands within a binding specificity class have distinct, but overlapping expression. These data suggest tissue- and stage-specific ligand–receptor interactions, and that although ligand and receptor expression is in many cases complementary, there are also overlaps. We discuss how the expression patterns of ligands for Eph-related receptors might relate to a general role in regulating the migration of cells and/or axons.

MATERIALS AND METHODS

RT-PCR using degenerate oligonucleotides. Total RNA was isolated from dissected hindbrains of 9.5-day postcoitum (dpc) mouse embryos or intact embryos of the same age according to the method of Chirgwin *et al.* (1979). This RNA was used as the tem-

plate for a reverse transcription reaction using either random hexamers or oligo-dT followed by PCR amplification with degenerate oligonucleotides. The degenerate oligonucleotides were fully redundant and were designed to amplify a fragment of approximately 200 bp which lay between two regions of conservation between B61 and Ehk1-L. The 5′ oligonucleotide consisted of a mixture corresponding to the peptides V/L D I I/Y C P and V/L D I I/Y C P H Y while the 3′ oligonucleotide encoded the peptide E K F Q R F/Y and these included an *Eco*RI site and a *Bam* HI site, respectively, to facilitate cloning of the PCR fragments. PCR was carried out in two steps, with 4 cycles of 94°C for 1 min, 42°C for 2 min, 68°C for 1 min, 72°C for 1 min, followed by 36 cycles of 94°C for 1 min, 60°C for 2 min, 68°C for 1 min, 72°C for 1 min. PCR products were sized fractionated on an agarose gel, subcloned into the pBluescript vector (Stratagene), and sequenced to confirm their identity as ligand family members.

cDNA library screening. An 8.5-dpc mouse embryo library constructed in lambda Zap II (kindly provided by Dr. John Gerhart) was screened at a stringency of 1× SSC, 0.1% SDS at 60°C to obtain cDNA's corresponding to the PCR fragments. Positive clones were transformed into pBluescript SK according to the manufacturer's instructions (Stratagene) and then characterised by sequence analysis.

DNA sequencing and analysis. Double-stranded DNA was sequenced by the dideoxy method using a T7 sequencing kit (Pharmacia). T3 and T7 primers were used to sequence the 200-bp PCR fragments and for end-sequencing cDNAs. cDNAs were sequenced in both directions using the TN1000 transposon method (Morgan *et al.*, 1996) with any gaps completed using sequence-specific oligonucleotides. Sequences were analysed using GCG (Madison, WI) and DNASTAR sequencing packages (Madison, WI).

RT-PCR using AL-1/RAGS-specific oligonucleotides. Total RNA was isolated from 8.5-, 9.5-, 10.5-, and 11.5-dpc embryos and used as the template for reverse transcription using oligo-dT followed by PCR amplification with AL-1/RAGS-specific oligonucleotides flanking the potential alternatively spliced exon. The 5′ oligonucleotide corresponded to the sequence 5′-GCCGAGAGTATTTCTACATCTCCTCTGC-3′ and the 3′ oligonucleotide to 5′-GACTGTGCTATAATGTCAAAACATCG-3′. The expected sizes of the PCR products made using the long and short AL-1/RAGS mRNAs as templates were 300 and 220 bp, respectively.

Southern blot analysis. Southern blot analysis was performed essentially as described by Sambrook *et al.* (1989). PCR products were separated by electrophoresis on a gel consisting of 3% NuSieve agarose (FMC Bioproducts). After blotting, the nylon membrane (Dupont) was probed with the ~700-bp coding region of AL-1/RAGS and washed to a stringency of 0.1× SSC, 0.1% SDS at 70°C.

In situ hybridization of whole mount and sectioned embryos. Mouse embryos were staged according to Kaufman (1992). Whole mount *in situ* hybridisation was performed as described (Nieto *et al.*, 1995). Anti-sense and sense riboprobes were produced by linearization of plasmid with appropriate restriction enzymes, followed by *in vitro* transcription from the T7 or T3 promoters in the presence of digoxygenin-11-UTP. Hybridisation of the probe to the cellular RNA was visualized using an alkaline phosphatase conjugated anti-digoxigenin antibody. Embryos were mounted on agarose, or in a depression slide, or flat mounted under a coverslip, and photographed using a Leica stereomicroscope. *In situ* hybridisation of sectioned embryos (15 μm) was performed as described by Schaeren Wiemers and Gerfin Moser (1993). Sections were photographed using an Axiophot microscope.

Whole mount staining using Fc tagged ligands and receptors. Whole mount staining of embryos using IgG-Fc-tagged ligands and receptors was performed essentially as described by Gale *et al.* (1996a). After removal of the extra-embryonic membranes, embryos were preblocked in 10% sheep serum, 2% bovine serum albumin in phosphate-buffered saline (PBS). Embryos were then incubated overnight at 4°C with ligand- or receptor-Fc at 5 μg/ml in 0.5× blocking solution. Embryos were repeatedly washed with PBS and then fixed in 4% paraformaldehyde (PFA). To inactivate endogenous phosphatases, embryos were incubated at 70°C for 1 hr. Embryos were again preblocked in 0.5× blocking solution plus 0.1% Triton-X 100 followed by incubation overnight at 4°C in a 1/1000 dilution of alkaline phosphatase-conjugated goat anti-human antibody (Promega). After extensive washing in Tris-buffered saline (TBS) plus 0.1% Triton X-100, embryos were transferred to alkaline phosphatase buffer (100 m*M* Tris–Cl, pH 9.5; 100 m*M* NaCl; 5 m*M* $MgCl_2$), including the substrates NBT (0.34 mg/ml) and BCIP (0.18 mg/ml) (Gibco BRL). Following colour development embryos were rinsed in PBS, fixed in 4% PFA, and photographed as described above.

RESULTS

Cloning of Ligands for Eph Family Receptors

Amino acid sequence comparisons revealed that B61 and Ehk1-L, members of the GPI-linked subclass of ligands, have several short regions of strong similarity, whereas the transmembrane ligand, Elk-L, is more divergent (Davis *et al.*, 1994). These ligands were cloned from the rat and human, so we set out to clone mouse orthologues and identify novel family members expressed during early development. Redundant oligonucleotides were designed corresponding to two regions of strong similarity between B61 and Ehk1-L (see Materials and Methods). After PCR amplification of 9.5-dpc mouse embryo cDNA with these oligonucleotides, a band of the predicted size, 200 bp, was obtained which was then purified and cloned. Sequencing of 42 clones identified 38 with significant similarity to members of the ligand family, and these corresponded to five distinct genes. Sequence comparisons suggested that we have isolated the mouse orthologues of B61 and Ehk1-L, and of three ligands reported while this work was in progress: ELF-1 (Cheng and Flanagan, 1994), AL-1/RAGS (Drescher *et al.*, 1995; Winslow *et al.*, 1995), and LERK4 (Kozlosky *et al.*, 1995). Full-length cDNA clones corresponding to four (B61, AL-1/RAGS, ELF-1, and LERK4) of these five clones were isolated from an 8.5-dpc mouse embryo cDNA library but we have been unable to isolate an Ehk1-L clone from this library. As expected this PCR strategy did not identify the more divergent Elk-L, and so a full-length mouse orthologue of this ligand was obtained by screening the mouse embryo cDNA library with a rat Elk-L probe.

Sequencing of the entire coding regions confirmed that we have isolated the mouse orthologues of B61, LERK4, and AL-1/RAGS (Figs. 1A and 1B). We end-sequenced ELF-1 and Elk-L, for which the sequence of mouse orthologues had been reported (Cheng and Flanagan, 1994; Shao *et*

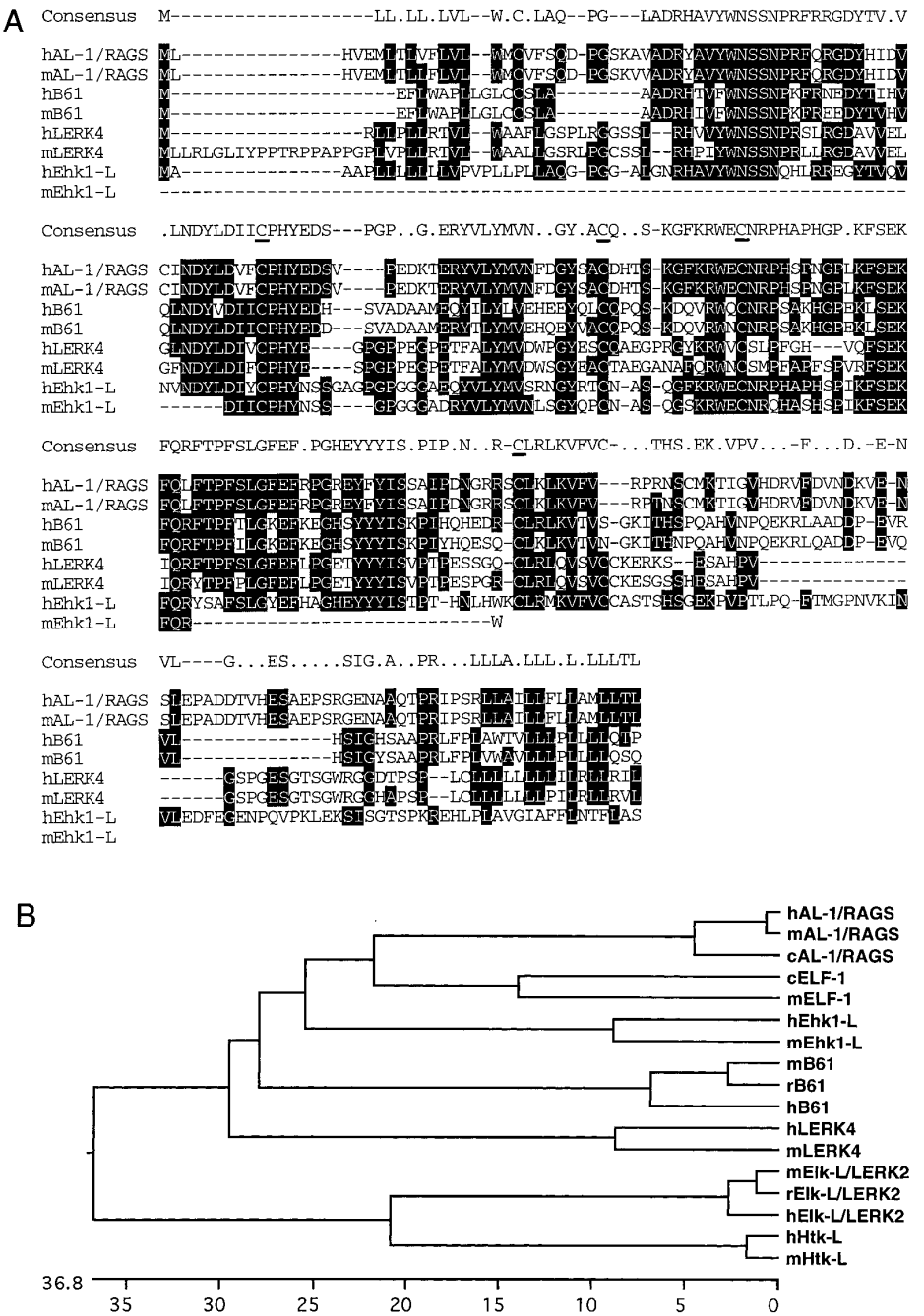


FIG. 1. Amino acid sequence comparison of ligands. (A) The deduced amino acid sequence of mAL-1/RAGS, mB61, mLERK4, and mEhk1-L is shown aligned with the human orthologues. Identity between ligand family members is indicated by shading and reported as the consensus. The four conserved cysteines (Pandey *et al.*, 1995a) are underlined. (B) Tree alignment of the seven Eph-family ligand members in various species. Ligands with a transmembrane domain are more homologous to each other, as are the GPI-anchored ligands to each other. c, chick; h, human; m, mouse; r, rat.

al., 1994; Fletcher *et al.*, 1994) to determine that they contained the entire coding region. Sequence comparisons showed that amino acid identity was very high between orthologous ligands in the mouse and human: 99% for AL-1/RAGS; 86% for B61; 82% for LERK4; and 81% for the PCR fragment of Ehk1-L.

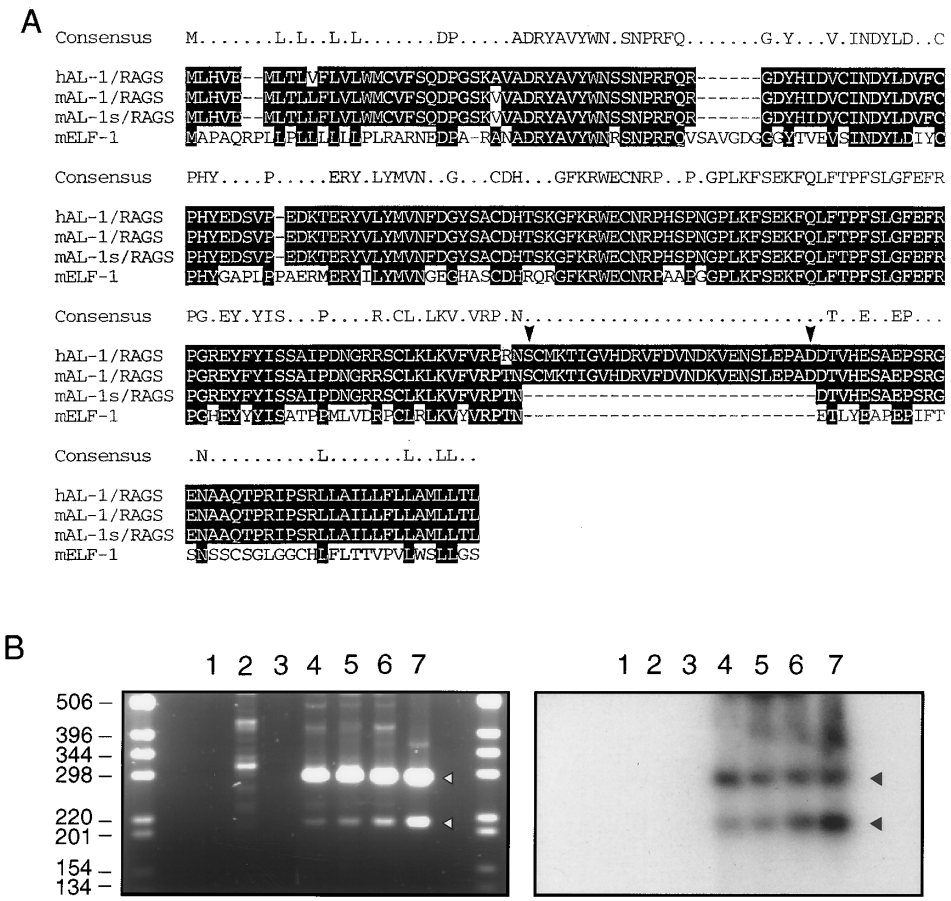


FIG. 2. Alternatively spliced transcripts of AL-1/RAGS. (A) The deduced amino acid sequence of human AL-1/RAGS is compared to the long (mAL-1/RAGS) and short (mAL-1s/RAGS) mouse orthologue and to mouse ELF-1. Identity between cDNAs is indicated by shading, whereas identity between all four cDNAs is reported as the consensus. The 27 a.a. exon missing in the short form of mAL-1/RAGS and from mELF-1 is indicated by the arrowheads. (B) Ethidium bromide-stained agarose gel (left) showing two RAGS transcripts (indicated by the white arrowheads) of 300 and 220 bp generated by RT-PCR. Lanes 1–3 show controls consisting of the 5′ and 3′ oligonucleotides with no DNA (lane 1); the 5′ oligonucleotide alone plus template (lane 2); and the 3′ oligonucleotide alone plus template (lane 3). Lanes 4–7 show the PCR products after using the 5′ and 3′ oligonucleotides to amplify cDNA templates made from embryos of 8.5 dpc (lane 4); 9.5 dpc (lane 5); 10.5 dpc (lane 6); and 11.5 dpc (lane 7). Size markers are located on each end of the gel with the sizes in bp indicated on the left. The figure on the right is the Southern blot of the agarose gel probed with the RAGS cDNA. The two RAGS-specific RT-PCR products corresponding to those bands in the ethidium bromide-stained gel are indicated by the arrowheads.

Two clones of AL-1/RAGS cDNA were isolated which were identical in DNA sequence except that the smaller clone was missing 81 bp corresponding to a 27 amino acid sequence within the C-terminal half of the protein. These 81 bp may represent an alternatively spliced exon, and this possibility is strengthened by the observation that alignment of the human and mouse AL-1/RAGS proteins with the mouse ELF-1 protein showed that the corresponding amino acid sequence was also absent in the ELF-1 protein (Fig. 2A). To analyse the expression of this alternatively spliced transcript we carried out PCR with primers flanking the alternative exon (Fig. 2B), and this confirmed that the shorter transcript is expressed in the 8.5–11.5 dpc embryo.

Comparison of Ligand Expression Patterns during Early Embryogenesis

We analysed the expression patterns of B61, LERK4, ELF-1, AL-1/RAGS, and Elk-L during mouse embryogenesis by whole mount *in situ* hybridisation. Partial descriptions have been reported of B61 (Shao *et al.*, 1995; Takahashi and Ikeda, 1995), Elk-L (Bouillet *et al.*, 1995), ELF-1 (Cheng and Flanagan, 1994; Cheng *et al.*, 1995), and AL-1/RAGS (Drescher *et al.*, 1995) expression at later stages, so for these we focus upon undescribed sites of expression and overlaps and differences between their expression domains and those of other ligands. The results of *in vitro* binding studies and the whole mount detection of interacting receptors and li-

gands with Fc fusion reagents (Gale *et al.*, 1996a) raised the possibility that members of a binding specificity class are functionally equivalent, and therefore it was important to compare the expression domains of GPI-anchored ligands with each other and with the expression of the entire class of ligands and potential target receptors. Similarly, we compared the expression of Elk-L with that of receptors and ligands in the same specificity class. Below, we first describe the major features of ligand expression at 8–10 days of development and then compare these with the patterns of receptor and ligand proteins detected by binding of Fc fusion proteins. This is followed by more detailed descriptions of the expression of ligands in specific tissues.

Expression of ligands. ELF-1 expression was first detected in the 8-dpc (6 somite) embryo in the dorsal region of the presumptive midbrain (Fig. 3A), and by ~9 dpc expression occured at high levels in the midbrain and at lower levels in the dorsal anterior hindbrain (Fig. 3B). At this stage expression in the midbrain appeared uniform, but by 10 dpc a posterior-to-anterior gradient has been established (data not shown), as described in the chick embryo (Cheng *et al.*, 1995).

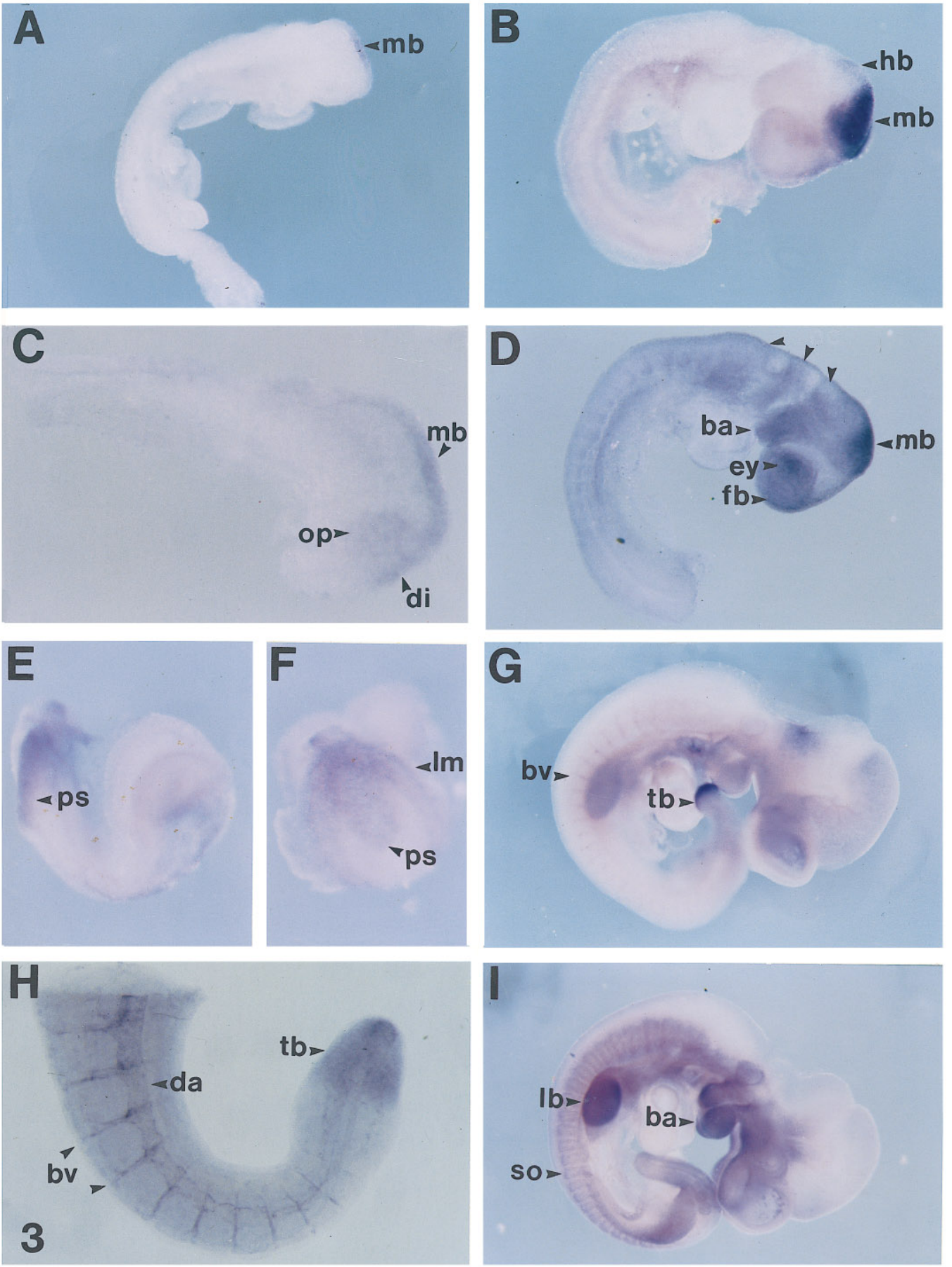
In the 8-dpc (6 somite) embryo, AL-1/RAGS expression was observed in the presumptive diencephalon, including the optic primordium, and in the midbrain (Fig. 3C). By 9 dpc there was a broad expression domain in the rostral forebrain, including the presumptive eyes and a dorsal stripe of expression in the diencephalon, and expression was up-regulated in the midbrain (Fig. 3D). In addition, lower levels of AL-1/RAGS transcripts were detected in the somites, hindbrain and in the branchial arches. Intriguingly, although the expression in the branchial arches appeared to correlate with neural crest streams, in particular the absence of expression at the crest-free regions adjacent to rhombomere 3 and ventral to the otic vesicle (Fig. 3D), analysis of sections revealed AL-1/RAGS transcripts only in the ectodermal component (Fig. 4A). By 10 dpc, a posterior-to-anterior gradient of AL-1/RAGS expression was detected in the midbrain (data not shown), similar to that reported in the chick embryo (Drescher *et al.*, 1995).

In contrast to ELF-1 and AL-1/RAGS, B61 and LERK4 expression was not detected in the CNS at these early stages. B61 expression was detected in the 8-dpc embryo in the primitive streak and lateral mesoderm (Figs. 3E and 3F) and at 9.5 dpc was strongly expressed in the tailbud and at lower levels in blood vessels (Figs. 3G and 3H). LERK4 expression was barely detectable at 8 dpc, but by 9.5 dpc occured in the ventral regions of the head, including the somites, the limb bud (Fig. 3I), and the ectoderm of the branchial arches (Figs. 3I and 4B). Localisation of the receptors which bind the GPI-linked class of ligands using a ligand-Fc fusion protein (Ehk1-L-Fc), revealed that the receptors are expressed in the mesenchyme of the 3rd branchial arch and absent from the ectoderm, as seen in coronal (Fig. 4C) and transverse section (Fig. 4D).

Elk-L was found to be expressed in a highly dynamic pattern during neurulation. In the 2–3 somite embryo a broad domain of expression was seen in the presumptive hindbrain and midbrain (Fig. 5A), which by the 4–5 somite stage had resolved into two domains, one in the presumptive anterior hindbrain and midbrain, and the other in presumptive r4, as ascertained by comparison with Eck (Sek-2), a marker of pre-r4 (Fig. 5B, and data not shown). Similar domains of expression were detected in the 7 somite (~8 dpc) embryo (Fig. 5C), then, at the 7–8 somite stage a downregulation of expression occured in presumptive r1 such that there were now three stripes of expression corresponding to pre-r4, pre-r2, and the midbrain (Fig. 5D). The width of the presumptive r2 stripe varied between embryos suggestive of a dynamic regulation, and by the 12 somite stage (8.5–9.0 dpc) Elk-L expression was restricted to rhombomere boundaries and occured at high levels in the dorsal midbrain and r1 (Fig. 5E). By 9.5–10 dpc Elk-L had been up-regulated in the roof plate, with weaker expression occuring in the hindbrain, dorsal diencephalon, and telencephalon, but had been down-regulated in the midbrain (Fig. 5F). In addition, expression occured in the branchial arches, the limbs, and the ventral part of the somites.

Overall, these data revealed that while there were distinct aspects of the expression of these ligands there were also overlaps. In the central nervous system (CNS), ELF-1 and AL-1/RAGS expression overlapped in the midbrain, although AL-1/RAGS expression was broader and also occured in the adjacent regions of the neural epithelium. At these early stages, overlapping expression of Elk-L, LERK4, ELF-1, and AL-1/RAGS occured in the limb bud. In addition, ligand expression occured in the somites, with LERK4 expressed throughout each somite, whereas Elk-L appeared restricted to presumptive dermamyotome and at later stages AL-1/RAGS expression appeared to be restricted to the myo-

FIG. 3. Comparison of GPI-anchored ligand expression patterns in early mouse embryos. Whole mount *in situ* hybridisation of mouse embryos was carried out to detect the expression pattern of 4 GPI-anchored ligands. Whole embryos are shown in lateral view unless otherwise indicated. (A and B) ELF-1 expression in a 6-somite embryo (A) and 9-dpc embryo (B). (C and D) AL-1/RAGS expression in the head of a 6-somite embryo (C) and a 9-dpc embryo (arrowheads mark apparent streams of expression) (D). (E–H) Expression of B61 mRNA in a gastrulation-stage embryo (~8 dpc) shown in lateral view (E) and caudal view (F) and in a 9.5-dpc embryo (G) with a higher magnification of the tail (H). (I) Expression of LERK4 mRNA in a 9.5- to 10-dpc embryo. Abbreviations for Figs. 3–9: ba, branchial arch; bv, blood vessel; da, dorsal aorta; dh, dorsal horn; di, diencephalon; dm, dermamyotome; drg, dorsal root ganglia; ec, ectoderm; el, ependymal layer; ey, eye; fb, forebrain; fp, floor plate; hb, hindbrain; iz, interdigital zone; lb, limb bud; lm, lateral mesoderm; mb, midbrain; me, mesenchyme; ml, mantle layer; mn, motor neuron; op, optic placode; ps, primitive streak; r2 and r4, presumptive rhombomere 2 and 4; r5, rhombomere 5; rp, roof plate; so, somite; tb, tailbud; te, telencephalon; tn, tendon; vh, ventral horn.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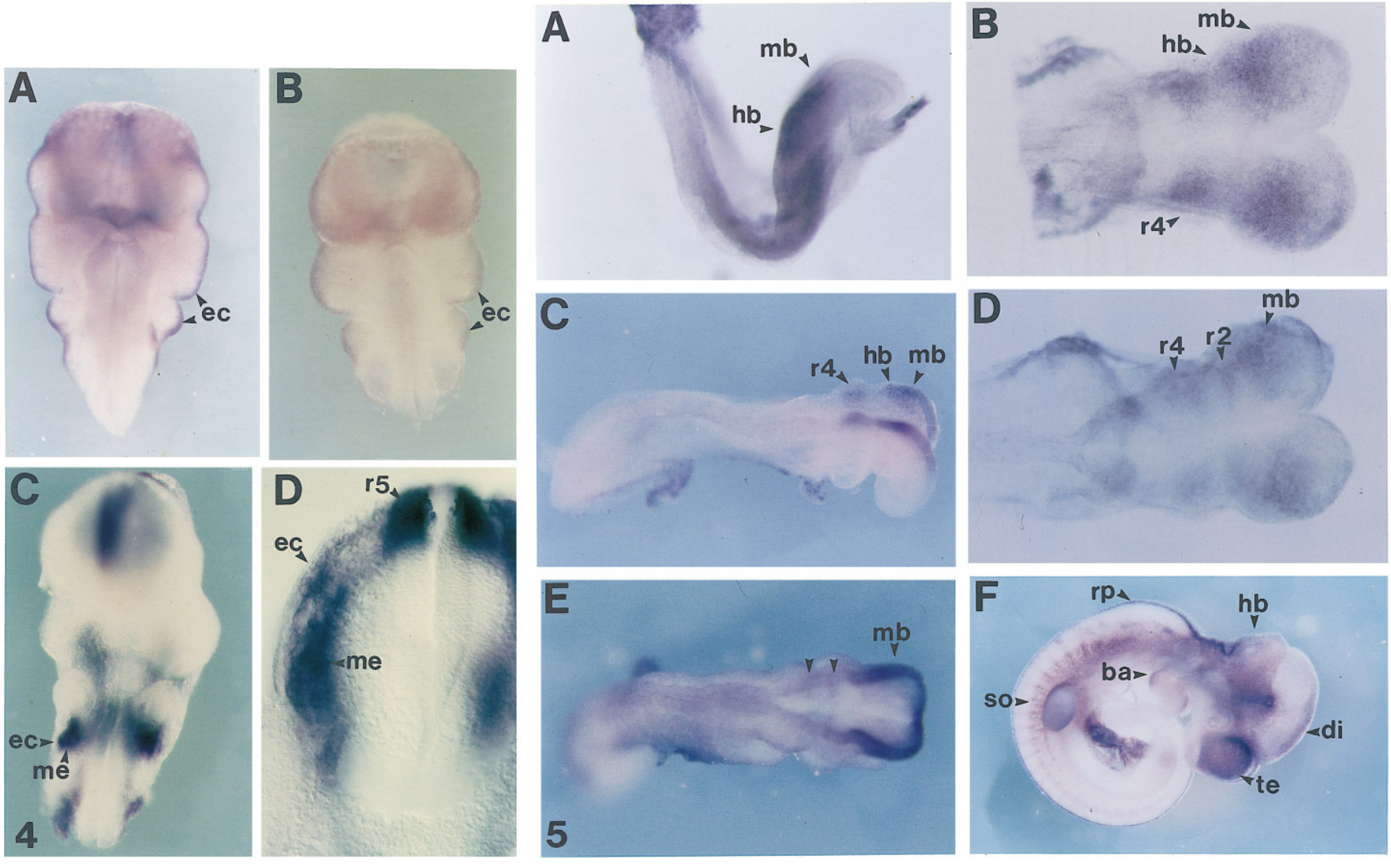


FIG. 4. Localisation of ligand and receptor expression in branchial arches. (A and B) Whole mount *in situ* hybridisation of 9.5-dpc embryos that have then been cut coronally to reveal expression of AL-1/RAGS (A) and LERK4 (B) in the ectoderm of the branchial arches. (C and D) 9.5-dpc embryos stained with the Ehk1-L-Fc fusion protein revealing localisation of the receptors which bind the GPI-anchored ligand subclass. Embryos have been cut coronally through the branchial arches (C) or in transverse section at the level of branchial arch 3 (D). For abbreviations see Fig. 3 legend.

FIG. 5. Elk-L expression. Whole mount *in situ* hybridisation depicting the expression pattern of Elk-L in the developing embryo. (A) Lateral view of 7.5-dpc embryo; (B) flat mount of 4- to 5-somite embryo; (C) lateral view of 7-somite embryo; (D) flat mount of 7- to 8-somite embryo; (E) dorsal view of 9-dpc embryo (arrowheads indicate expression at rhombomere boundaries); (F) lateral view of 9.5- to 10-dpc embryo. Flat mounting was carried out such that the dorsal/lateral edge is lateral and the ventral/medial midline is medial. Embryos are positioned to highlight cranial expression patterns where anterior is to the right and posterior is to the left. For abbreviations see Fig. 3 legend.

tome (not shown). Finally, Elk-L, LERK4, and AL-1/RAGS were expressed in the branchial arches and ELF-1 appeared to have low level expression in this region.

Expression of ligand and receptor classes. To compare these data with the expression of classes of receptors and ligands we stained 9–9.5 dpc embryos with a number of ligand- and receptor-Fc fusion reagents: Ehk1-L, AL-1/RAGS, LERK4, all in the GPI-anchored ligand specificity class, and the corresponding Ehk1 and Sek-1 receptors, and the transmembrane ligand, Elk-L, and the corresponding Nuk receptor. Reagents from the same specificity class detect similar patterns, but there is some variation in the relative intensity of staining of tissues that presumably reflect the quantitative differences in binding to distinct target receptors or ligands (Gale *et al.*, 1996a). Although Sek-1-Fc has been shown to bind transmembrane ligands in assays of transfected cells, we do not detect binding to known sites of embryonic expression of these ligands (see below). One possible explanation is that the whole mount staining method only detects the highest affinity interactions.

Ehk1-L, AL-1/RAGS, and LERK4 fusion proteins detect receptors expressed in mesoderm in the tail bud, the dorsal part of the definitive somites (weaker for LERK4), the third branchial arch (weaker for AL-1/RAGS), hindbrain, diencephalon, and in the most anterior part of the telencephalon (broader for LERK4) (Figs. 6A, 6C, 6E, and 6F). In addition, LERK4-Fc detects receptor in the newly forming somites (Fig. 6E), but these are not detected by Ehk1-L-Fc or AL-1/RAGS-Fc. A dynamic regulation of receptor in the branchial arches is detected by LERK4-Fc, with expression occuring in the mesenchyme of the third branchial arch in the ~23 somite embryo, which by ~29 somites has been down-regulated and receptor is now detected in the anterior part of the first branchial arch and the posterior part of the second arch. As found at later stages of development (Gale *et al.*, 1996a), these receptor expression domains are complementary to those of the corresponding GPI-anchored ligand class detected by Ehk1 and Sek-1 in the ventral part of the somites (weaker for Sek-1), the first, second, and fourth branchial arches, midbrain, roofplate, eye, and in the telencephalon excluding the most anterior region (Figs. 6B and 6D). Comparing the expression domains of this ligand class with individual GPI-anchored ligands, there is a clear correlation with the expression of ELF-1 and AL-1/RAGS in the midbrain and AL-1/RAGS in the eye, which are therefore complementary to the expression of potential receptors. However, AL-1/RAGS and LERK4 transcripts are detected in all of the branchial arches and throughout the somites, whereas the Sek-1- and Ehk1-Fc reagents only detect members of this ligand class in subsets of these tissues.

Detection of receptors with Elk-L-Fc reveals expression in mesoderm in the tailbud and the anterior part of the somites (Fig. 6G), whereas detection of transmembrane ligands with Nuk-Fc reveals expression in the posterior half of the somites, in the hindbrain, in the posterior midbrain, the roof plate, and in the clefts between the first and second, and the second and third branchial arches (Fig. 6H). Nuk-Fc therefore detects ligands that correlate with Elk-L expression in the roof plate and in the clefts at the anterior part of the first and posterior half of the second branchial arch, and presumably is detecting other transmembrane ligands elsewhere.

Expression of Ligands in Limbs

Expression of all five ligands was detected in developing limbs. Elk-L and B61 had very distinct and dynamic expression patterns which are described in detail, whereas ELF-1, LERK4, and AL-1/RAGS were expressed in similar, less dynamic patterns and are presented together.

Elk-L expression. In the 10-dpc embryo, Elk-L expression was detected in the distal posterior region of the forelimb bud (Fig. 7A). This expression domain then expanded such that at around 10.5 dpc staining remained stronger in the posterior region but now encompassed most of the distal limb bud (Fig. 7B). By approximately 11 dpc, as the limb becomes plate-shaped, Elk-L expression was down-regulated such that low levels persisted in the distal edge. In addition, an arc of expressing cells was observed orthogonal to the proximal–distal axis, that was broad at the anterior edge and narrowed towards the centre of the limb (Fig. 7C). Elk-L expression also occured in the central region of the limb just proximal to this arc of cells. By 11.5 dpc the thin arc of cells had now lengthened such that it spanned the A-P axis of the hand/foot plate (Fig. 7D) and the proximal expression domain appeared to have separated into two longer stripes. At 12–12.5 dpc two oval domains of expression were seen on the ventral side of the limb at the base of the paddle which may correspond to two sites of chondrogenesis (Fig. 7F). On the dorsal side there was an up-regulation of expression over the whole of the proximal forelimb with radiating rays which may correspond to the forming digits (Fig. 7E). The expression domain on the edge of the hand/foot plate remained, but was down-regulated in the interdigital zone (Fig. 7E). Finally, at approximately 14 dpc, expression was seen over the entire ventral hand/foot plate and appeared to outline developing tendons and also occured at the tip of the digits (Figs. 7G and 7H). This latter expression encompassed both the dorsal and ventral surfaces at 15.5 dpc (data not shown) and may correspond to the developing nail beds.

B61 expression. At 10.5 dpc there was no expression of B61 in either the forelimb or the hindlimb (data not shown). However, by approximately 11.0–11.5 dpc B61 expression was detected in the forelimb, whereas a signal was just detectable in the hindlimb. At this stage strong expression was seen in a circular domain in the posterior-distal part of the developing hand plate and lower level expression occured in a complex network, especially prominent along the midline of the proximal-distal axis, suggestive of blood vessels (Fig. 8A). When a late 11.5-dpc hindlimb was viewed in cross-section the circular expression domain was seen to be located in the mesoderm in the middle of the dorsal/

ventral plane (Fig. 8C) while the fainter expression domain lay closer to the surface ectoderm (data not shown). By 12.0 dpc the strong expression site in the hindlimb had elongated into a curved rod extending from the distal posterior edge and towards the centre (Fig. 8B). By 12.5–13.0 dpc this expression domain had disappeared in the hindlimb. At this time expression was detected on the ventral side of the hand/foot plate in a pattern that correlates with the blood sinuses and sites of future blood vessels (Fig. 8D).

Expression of ELF-1, LERK4, and AL-1/RAGS. In the 9.5- to 10-dpc embryo ELF-1, LERK4, and AL-1/RAGS were broadly expressed over the limb bud (Fig. 3I and data not shown) but in older limbs expression was more localized. At 12 dpc ELF-1 expression was detected in the interdigital zone and at the tips of the newly forming digits, but was absent over the central portion of the digit (Fig. 8E). By approximately 13 dpc the interdigital zone expression was down-regulated and expression was found only around the edges of the future digits (Fig. 8F). Expression of LERK4 was detected in the 12.5-dpc limb over the proximal limb and hand/foot plate. As for ELF-1, expression appeared higher around the forming digits but, for LERK4, was restricted to the proximal end of the digit (Fig. 8G). AL-1/RAGS was also expressed in the interdigital zone in the ~12 dpc embryo limb (data not shown) and by 12.5–13 dpc, strong expression was detected over the proximal limb while expression in the hand/foot plate was very weak. However, like ELF-1 and LERK4, faint expression could be detected around the edges of the forming digits (Fig. 8H).

Expression of Ligands in the Spinal Cord

Expression of three ligands, Elk-L, LERK4, and AL-1/RAGS, was detected in cross-sections of the anterior spinal cord. Elk-L expression was observed in the 9.5-dpc embryo on the dorsal aspect of both sides of the open neural tube and then as a single stripe where the neural tube had fused to form the roof plate down the length of the embryo (Fig. 5F). In a cross-section of the anterior spinal cord of a 13.0-dpc embryo Elk-L expression was detected in the floor plate, the ependymal layer (ventricular zone) at higher levels dorsally than ventrally, as well as in the dorsal root ganglia (Fig. 9A). At this same stage LERK4 was strongly expressed in the ependymal layer with a lower level of expression in the mantle layer, and with higher levels ventrally in both of these regions (Fig. 9B).

In the 11.5-dpc embryo AL-1/RAGS expression was seen in the most anterior spinal cord in a small patch in the ventricular zone (Fig. 9C). In the 13-dpc embryo strong AL-1/RAGS expression was detected in a subset of cells in the ventral horn, with a low level of signal occuring more broadly, as well as in the dorsal horn (Fig. 9D). To ascertain the identity of the strongly expressing cells in the ventral horn we compared AL-1/RAGS expression (Fig. 9E) with Islet-1 (Fig. 9F), which in ventral regions is a specific marker of motor neurons (Tsuchida *et al.*, 1994). This revealed that AL-1/RAGS transcripts are expressed in a subset of motor neurons.

DISCUSSION

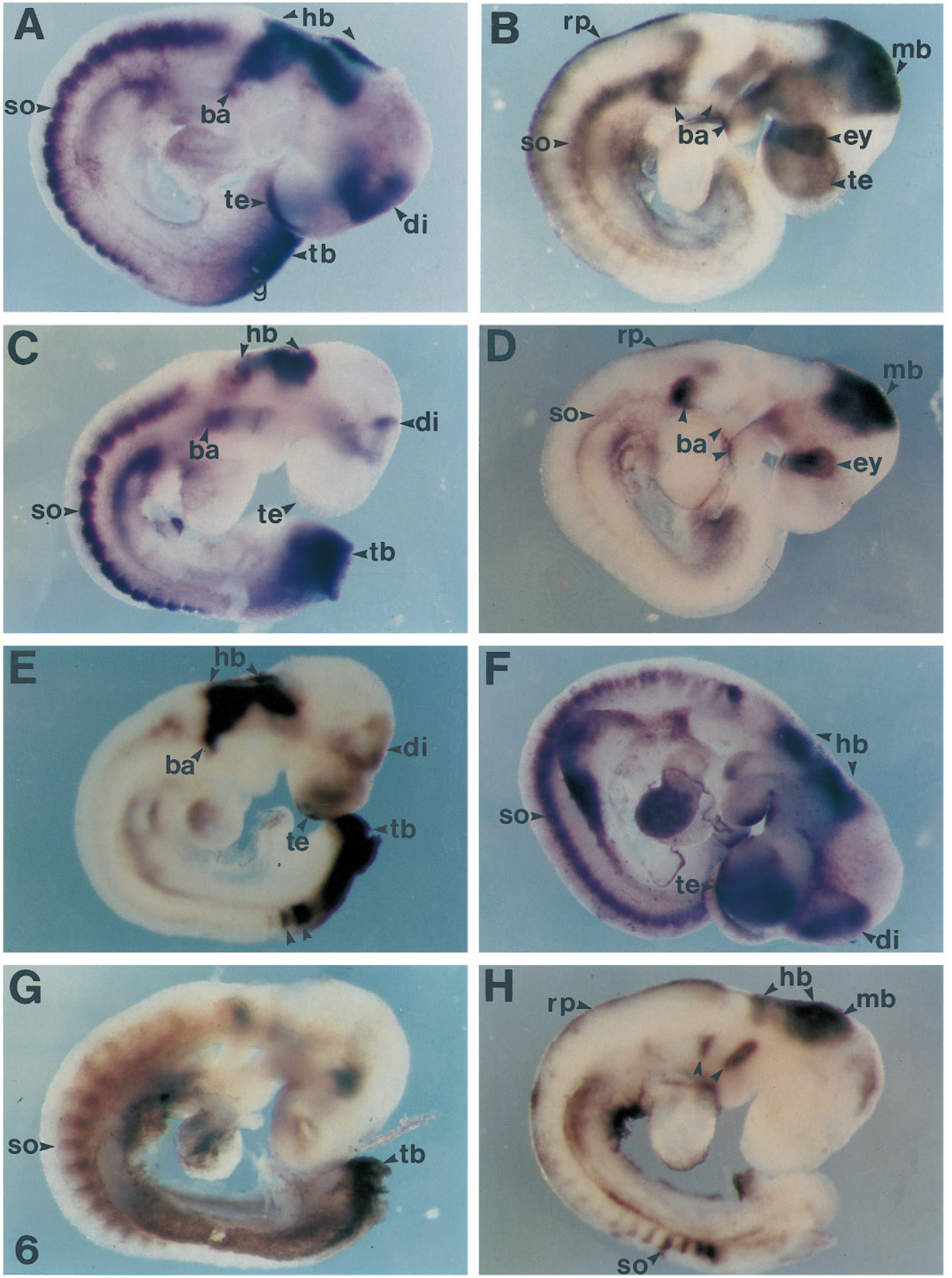
In this study, we cloned members of the family of ligands for Eph-related receptors from the mouse embryo and characterised their developmental expression patterns. Below, we first discuss the cloning and sequence of ligands, then discuss the relationships between the expression patterns of ligands and of potential target receptors, and finally, the implications for their potential developmental functions.

Cloning and Sequence of Mouse Ligands

Our PCR-based strategy was based upon sequences conserved between B61 and Ehk1-L, and in addition to amplifying mouse orthologues of these ligands, we identified three ligands which were independently cloned by other groups based on their binding to the extracellular domain of Eph family receptors: ELF-1 (Cheng and Flanagan, 1994), LERK4 (Kozlosky *et al.*, 1995), and AL-1/RAGS (Winslow *et al.*, 1995; Drescher *et al.*, 1995). Although it is noteworthy that these different approaches have identified the same ligands, it is possible that further GPI-linked ligands exist that have not been identified in our screen, due to sequence divergence at the region corresponding to our oligonucleotides and/or because of low or no expression in the 9.5-day mouse embryo.

We identified two variants of mouse AL-1/RAGS cDNA that differ by an 81-bp sequence corresponding to 27 amino acids within the C-terminal half of the encoded polypeptide. This is the first indication of alternative splicing that can generate distinct isoforms of members of this ligand family and provides a partial explanation for the observation of three sizes of human AL-1/RAGS protein (Winslow *et al.*, 1995). The longer isoform of AL-1/RAGS repels retinal axons (Drescher *et al.*, 1995) and causes the bundling of cortical axons by binding to the REK7 receptor (Winslow *et al.*,

FIG. 6. Distribution of GPI-anchored and transmembrane ligands and their receptors. The expression of receptor and ligand specificity classes in 9- to 9.5-dpc embryos was detected with GPI-anchored ligands (Ehk1-L-Fc, AL-1/RAGS-Fc, LERK4-Fc) and their corresponding receptors (Ehk1-Fc, Sek-1-Fc) or a transmembrane ligand (Elk-L-Fc) and a corresponding receptor (Nuk-Fc). Embryos were photographed in lateral view. (A) Ehk1-L-Fc at ~23 somites; (B) Ehk1-Fc at ~23 somites; (C) AL-1/RAGS-Fc at ~20 somites; (D) Sek-1-Fc at ~25 somites; (E) LERK4-Fc at ~23 somites (arrowheads indicate newly forming somites); (F) LERK4 at ~29 somites; (G) Elk-L-Fc at ~25 somites; (H) Nuk-Fc at ~20 somites (arrowheads indicate clefts between branchial arches 1 and 2, and 2 and 3). For abbreviations see Fig. 3 legend.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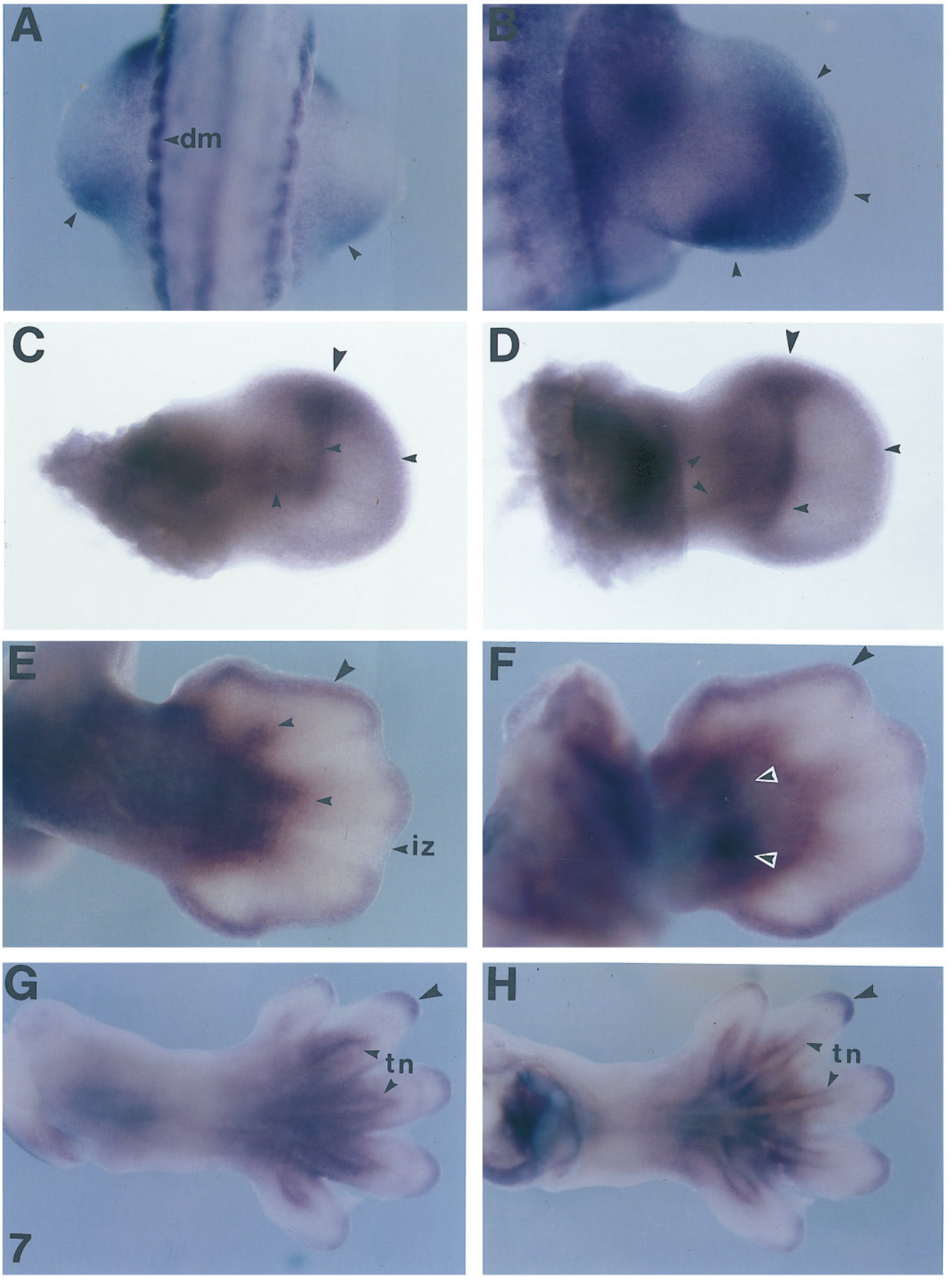
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1995), and it will be interesting to examine whether the shorter isoform identified here has a different binding specificity and biological effect. It is striking that the published mouse ELF-1 cDNA (Cheng and Flanagan, 1994) lacks a region precisely corresponding to this alternatively spliced sequence, and this raises the possibility that a longer isoform of ELF-1 exists. Analysis of this will be particularly interesting in view of the overlap in expression of ELF-1 and AL-1/RAGS in the developing tectum, suggestive of a possible overlapping or cooperative function in establishing the retinotectal map.

Complementary and Overlapping Expression of Ligands and Receptors

The detection with Fc fusion proteins of the GPI-anchored ligand class and the corresponding receptor class reveals complementary domains in many tissues at 10.5 dpc (Gale *et al.*, 1996a) and we find that although some aspects of the expression domains are different at 9.5 dpc, there is a similar complementarity. Consistent with these data, the expression domains of the receptor class in the forebrain, hindbrain, third arch neural crest, dorsal part of the somites, and early mesoderm correlate well with the superimposed expression patterns of Sek-1 (Nieto *et al.*, 1992) and Ebk (Ellis *et al.*, 1995). Similarly, the expression of the corresponding ligand class in the midbrain, and in the mesenchyme around the forming cartilage in the limb (Gale *et al.*, 1996a) correlates with the expression of ELF-1, AL-1/RAGS, and LERK4 in these tissues. However, other aspects of the ligand class expression domains do not correlate with the expression patterns of individual GPI-anchored ligand mRNAs: at 9.5 dpc LERK4 and AL-1/RAGS RNA are expressed in all branchial arches, whereas receptor-Fc detects ligands only in the 1st, 2nd, and 4th arch; LERK4 and AL-1/RAGS RNA are detected throughout the somites, whereas receptor-Fc detects ligands only in the ventral part; LERK4 is expressed in the ventral part of the ventricular zone of the spinal cord and AL-1/RAGS in the ventral horn, whereas receptor-Fc detects ligands only in the dorsal and medial regions (Gale *et al.*, 1996a). There are several possible explanations of why receptor-Fc is not detecting ligands in tissues in which ligand mRNA is present. First, that the receptor-Fc reagents are not detecting AL-1/RAGS or LERK4, but rather other ligands, yet to be cloned, that are expressed in complementary patterns with receptors, but this seems very unlikely in view of the high affinity of their *in vitro* binding. Second, that there is translational control such that ligand mRNA and protein expression do not correlate. Third, that ligand protein is masked or degraded such that it is not detected by receptor-Fc. Consistent with the latter explanation, in all cases binding of receptor-Fc to ligand is not detected in those places in which receptor protein is present, for example in the dorsal part of the somites and the third branchial arch. This would suggest that, where expression overlaps, the majority of ligand may be sequestered at sites of cell contact by an excess of receptor, and that soluble receptor-Fc may not be able to compete with endogenous membrane-bound receptor for binding to ligand. Alternatively, or in addition, ligands may be internalised upon binding to receptor as has been shown for brain-derived neurotrophic factor (BDNF) (Biffo *et al.*, 1995). These possibilities could be distinguished when appropriate antibodies to detect ligands are available. Although these observations point to a possible limitation of receptor-Fc fusion reagents, they provide evidence for a sequestering or internalisation that might be of functional significance.

In summary, our data suggest that in many tissues complementary expression of Eph-related receptors and ligands occurs, whereas in others there is overlapping expression, and it is pertinent to consider what the functional significance of this might be. On the one hand, the coexpression of B61 ligand and Eck receptor in endothelial cells has been implicated in stimulating the migration of these cells during angiogenesis (Pandey *et al.*, 1995b), and therefore it is possible that the receptors and ligands also have roles in promoting migration of other cells. On the other hand, opposing gradients of expression of AL-1/RAGS and ELF-1 ligands in the midbrain and Mek4 receptor in retinal axons may underlie the targeting of these axons by a repulsion mechanism (Cheng *et al.*, 1995; Drescher *et al.*, 1995). It is therefore possible that complementary expression reflects a role in restricting cell or axon migration in other tissues. Although restrictions to cell movement have only been reported in a few tissues, it remains possible that they are

FIG. 7. Elk-L expression in the limb. Whole mount staining of embryos by *in situ* hybridization to detect Elk-L expression in the developing limb. Limbs are viewed such that anterior is to the top; posterior is to the bottom; proximal is to the left; distal is to the right. The stages of the limbs are: (A) 10-dpc limb bud (arrowheads indicate posterior staining); (B) 10.5-dpc limb bud (arrowheads indicate expression in the distal mesenchyme); (C) 11-dpc limb bud (large arrowhead marks the extended anterior end of the arc while small arrowheads point out the perpendicular expression domains and expression in the distal edge of the limb bud); (D) 11.5-dpc limb bud (large arrowhead marks the extended anterior end of the arc domain while small arrowheads indicate expression at the distal edge of the limb bud and expression in the arcs running proximodistally and anteroposteriorly; (E) dorsal side of a 12- to 12.5-dpc limb (large arrowhead marks expression at the distal edge and small arrowheads highlight rays of expression in forming digits); (F) ventral side of a 12- to 12.5-dpc limb (large arrowhead marks expression at the distal edge and white arrowheads indicate expression domains at the base of the paddle); (G and H) dorsal and ventral sides, respectively, of 14-dpc limb (arrowheads indicate expression in tips of digits). For abbreviations see Fig. 3 legend.

more widespread than currently recognised, since if such restraints are partial they will only be detected by quantitative analysis of cell movement; even in the hindbrain, the clearest example of lineage restriction, the restriction is not absolute (Birgbauer *et al.*, 1995) and may act in parallel with community effects that regulate the identity of those cells that do cross boundaries.

Interactions between Multiple Ligands and Receptors

Previous studies of ELF-1 (Cheng and Flanagan, 1994; Cheng *et al.*, 1995) and AL-1/RAGS (Drescher *et al.*, 1995) have shown overlapping expression in the midbrain, and we find that such overlaps between ligands of the same specificity class also occur in other tissues, including the branchial arches and limbs. This overlapping expression of ligands, and also of receptors (Becker *et al.*, 1994), raises the possibility of cooperative or overlapping functions, and predicts that knockouts of individual genes may reveal only some aspects of their developmental function (Chen *et al.*, 1996).

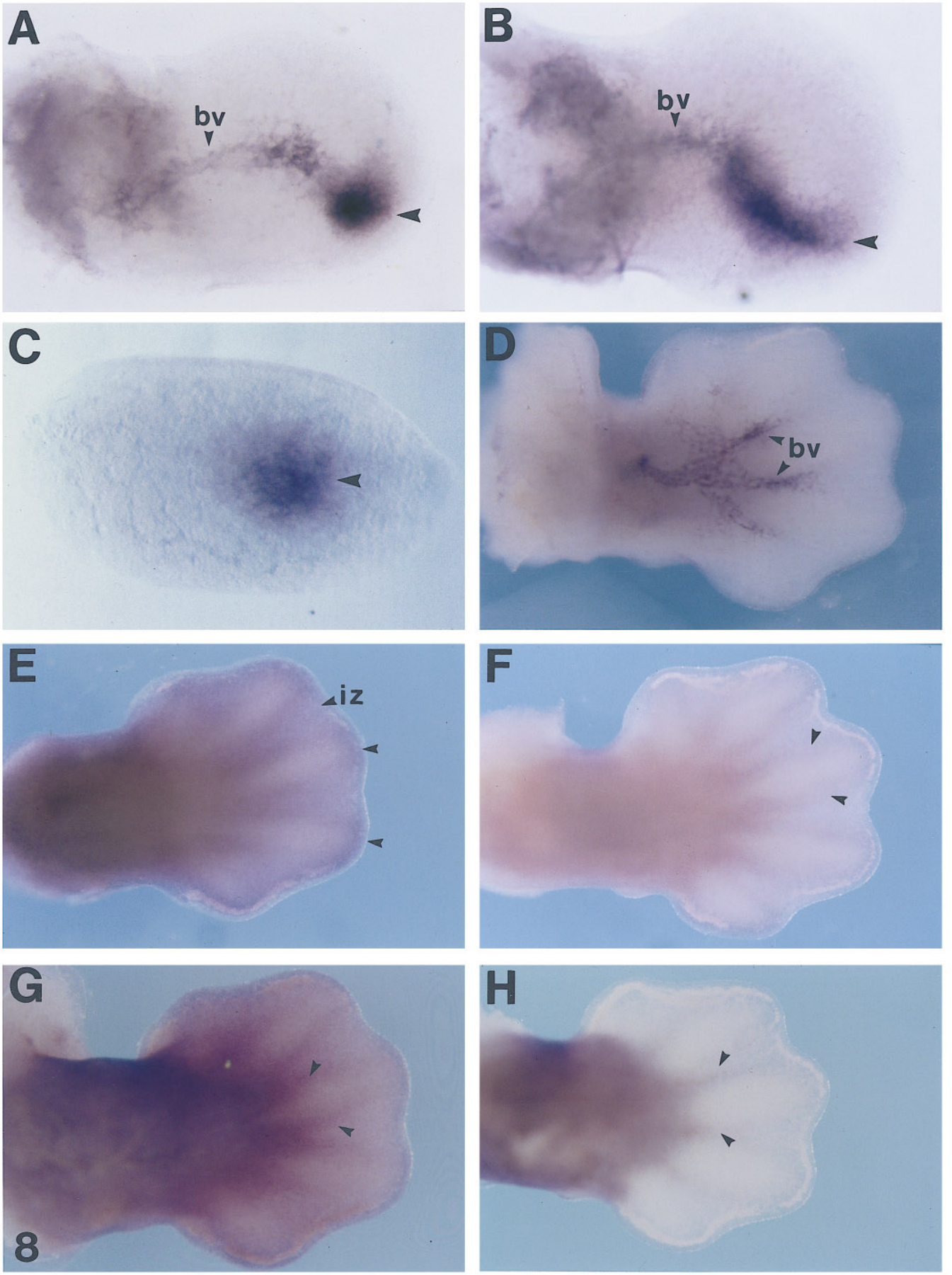
We do not find any simple correlations between the expression of one ligand with one target receptor, but rather our data suggest that a ligand may interact with different receptors in different tissues, and vice versa. For example, B61 and Eck are expressed in the epithilia of the lung and gut in the 19 dpc rat embryo (Shao *et al.*, 1995). However, Eck and B61 expression only partially correlate during early development, with overlapping expression in the primitive streak and the distal limb bud, but not in other tissues: Eck is also expressed in r4 and mesoderm adjacent to r4 (Becker *et al.*, 1994; Ganju *et al.*, 1994; Ruiz and Robertson, 1994), and B61 in lateral mesoderm, the tail bud, and blood vessels at 8.5–9.5 dpc. This raises the possibility that other members of the ligand and receptor families interact with Eck and B61 in these latter tissues, and that stage-specific interactions with receptors occurs in blood vessels. A similar situation may occur for ligands and receptors expressed in the hindbrain. We find that Elk-L expression is transiently restricted to r2 and r4, which overlaps with expression of Elk-L3 (Gale *et al.*, 1996b) and the more prolonged expression of ELF-2 in r2, r4, and r6 (Bergemann *et al.*, 1995; Flenniken, unpublished observations), and overlaps and temporal differences also occur for the receptors expressed in r3 and r5 that these ligands can interact with: Sek-1 is up-regulated early during formation of these rhombomeres, whereas Sek-3/Nuk and Sek-4 are up-regulated later. Sek-1 can interact with Elk-L3 and ELF-2, and Sek-3 and Sek-4 with Elk-L3, ELF-2, and Elk-L, and thus there may be stage-specific interactions between multiple receptors and ligands in this tissue.

Taken together, the results of *in vitro* binding and expression studies raise the possibility that receptors and ligands within the same binding specificity class are functionally equivalent, and that the existence of a large family only serves to enable complex patterns of developmental regulation (Brambilla and Klein, 1996; Gale *et al.*, 1996a). However, since there are differences in the relative binding affinities of receptors and ligands within the same class, it will be important to analyse whether this reflects some degree of specificity and functional divergence.

Potential Roles of Ligands during Early Development

Studies of the developmental expression and function of Eph-related receptors and ligands suggest that they have roles in tissue patterning and at later stages in neuronal pathfinding, and it is possible that both may involve a regulation of cell or axon migration. Many aspects of the expression described here seem likely to reflect roles in tissue patterning, either because expression is early and transient or does not correlate with the routes or targets of axons. For example, as found for several receptors (Nieto *et al.*, 1992; Becker *et al.*, 1994; Ganju *et al.*, 1994; Ruiz and Robertson, 1994; Xu *et al.*, 1994), a number of ligands are expressed early during mesoderm development: B61 in the primitive streak and lateral mesoderm, and later in the tail bud, LERK4 throughout the somites, and AL-1/RAGS and Elk-L in the dermamyotome. The expression in somites overlaps with several Eph-related RTKs, including Sek-1 (Nieto *et al.*, 1992), Sek-4 (Becker *et al.*, 1994), and Ebk (Ellis *et al.*, 1995), and it is interesting that the expression domains correlate with two distinct aspects of somite patterning. Certain ligands (AL-1/RAGS, Elk-L) and receptors (Ebk, Sek-1 in the mature somite) are expressed in different cell types along the dorsoventral axis of the somite, whereas the ELF-2 ligand (Bergemann *et al.*, 1995) and Sek-1 receptor (Nieto *et al.*, 1992) are expressed in posterior and anterior

FIG. 8. B61, ELF-1, LERK4, and AL-1/RAGS expression in the limb. Whole mount *in situ* hybridisation was carried out to detect expression of B61, ELF-1, LERK4, and AL-1/RAGS in the limb. Limbs are viewed such that anterior is to the top; posterior is to the bottom; proximal is to the left; and distal is to the right. (A–D) B61 expression in the 11- to 11.5-dpc limb bud (arrowhead marks distal circular domain) (A); 12-dpc limb (arrowhead marks distal tip of the curved rod domain) (B); cross-section through the circular domain (indicated by arrowhead) of the distal limb bud of an 11.5-dpc embryo (C); the ventral side of a 12.5- to 13-dpc limb (D). (E and F) ELF-1 expression as viewed from the dorsal side of a 12-dpc limb (arrowheads indicate expression at tips of digits) (E) and 13-dpc limb (arrowheads mark expression at edges of future digits) (F). (G) Dorsal view of a 12.5-dpc limb probed with LERK4 (arrowheads mark staining at edges of future digits). (H) 12.5- to 13-dpc limb probed with AL-1/RAGS (arrowheads mark expression at edges of future digits). For abbreviations see Fig. 3 legend.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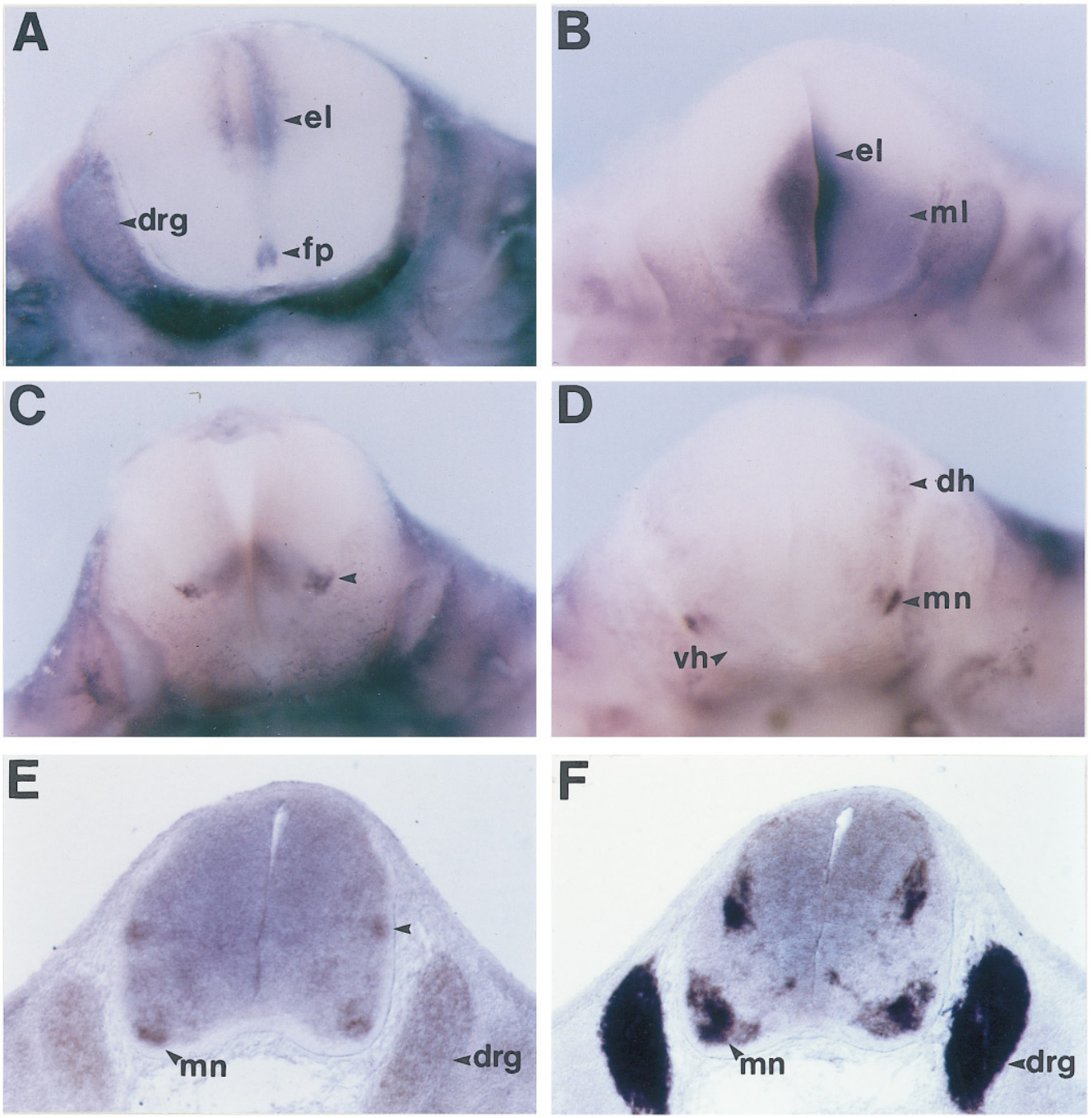

FIG. 9. Expression of Elk-L, LERK4, and AL-1/RAGS in the spinal cord. *In situ* hybridisation was carried out to detect ligand expression in the spinal cord and to compare expression with Islet-1. Transverse sections through the anterior spinal cord of embryos hybridised in whole mount revealed expression of (A) Elk-L in a 13-dpc embryo; (B) LERK4 in a 13-dpc embryo; (C) AL-1/RAGS in a 11.5-dpc embryo (the arrowhead indicates expressing cells in the ventricular zone; note that the more medial signal corresponds to the ventricular zone in deeper plane of focus); and (D) AL-1/RAGS in a 13-dpc embryo. *In situ* hybridisation to adjacent sections of a 12-dpc embryo at the level of the upper limb was carried out with (E) AL-1/RAGS probe and (F) Islet-1 probe. Expression of AL-1/RAGS was detected in motor neurons, as well as a more dorsal population (arrowhead). Islet-1 is a marker of motor neurons ventrally and also occurs in an unidentified dorsal population. For abbreviations see Fig. 3 legend.

halves of newly forming somites. This raises the question as to whether these might be involved in the migration or segregation of cells during somite formation and differentiation. Further examples, discussed below, of potential roles in tissue patterning rather than axon pathfinding are the neural epithelium and developing limb.

Spatial Patterning in the Neural Epithelium

Sek-1, Sek-2/Eck, Sek-3/Nuk, Sek-4, and Ebk have segmental expression in the hindbrain (Nieto *et al.*, 1992; Becker *et al.*, 1994; Henkemeyer *et al.*, 1994; Ruiz and Robertson, 1994; Ellis *et al.*, 1995), and we were therefore interested to analyse expression of ligands in this region. ELF-1 expression only occurs in the dorsal part of r1 and r2, so is unlikely to interact with Sek-1 in r3 and r5, though it does overlap with the transient, low-level expression of Sek-1 in r2 (Nieto *et al.*, 1992). None of the other GPI-anchored ligands are expressed in the hindbrain, and it remains unclear what ligand(s) interacts with Eck and Ebk in this tissue. However, our finding that the transmembrane ligand, Elk-L, is segmentally expressed in r2 and r4, indicates that this has complementary expression with the Sek-3 and Sek-4 receptors that it interacts with. Furthermore, as discussed above, Elk-L expression overlaps with Elk-L3 and ELF-2 in even-numbered rhombomeres, and Sek-3 and Sek-4 expression overlaps with Sek-1 in odd rhombomeres, and thus this complementarity occurs for multiple members of a binding specificity class. As a consequence, ligand–receptor interactions will then occur predominantly at rhombomere boundaries, and it may be significant that as Elk-L and Elk-L3 (Gale *et al.*, 1996b) transcripts are down-regulated, expression persists at boundaries. This raises the question as to the function of this complementary receptor and ligand expression. One possibility is that it relates to the formation of distinct boundary cells at the interface between odd- and even-numbered rhombomeres (Heyman *et al.*, 1993; Xu *et al.*, 1995). Other possibilities are suggested by functional studies of Sek-1. Dominant negative interference with Sek-1 function leads to the presence of cells with r3/r5 identity in even-numbered rhombomeres, suggesting a role in restricting the movement of cells from r3/r5 or in switching the identity of any cells that cross from odd to even territory (Xu *et al.*, 1995). Thus, a complementary expression of Sek-1 ligand could underlie a repulsion of r3/r5 cells by r2/r4/r6 or cause activation of Sek-1 which triggers a switch in identity of any r3/r5 cells that cross into r2/r4/r6. It will therefore be interesting to determine whether the complementary expression of Sek-3 plus Sek-4 and Elk-L, Elk-L3 plus ELF-2 reflects a role in either of these processes.

Previous work has shown that AL-1/RAGS and ELF-1 are expressed in a gradient in the midbrain that may underlie the pathfinding of retinal axons. We find that the midbrain expression of these genes is established in uniform domains in the early neural epithelium, long before axonogenesis. While it is possible that this early expression is of no functional significance, it is striking that it is complementary to the expression domains of receptors detected with ligand-Fc reagents at these stages. This raises the possibility that, as in the hindbrain, complementary expression of receptors and ligands could stabilise subdivisions of the neural epithelium. According to this, AL-1/RAGS and ELF-1 may initially stabilise their expression domain that later becomes the target of retinal axons.

Potential Roles of Ligands in the Branchial Arches

In addition to expression in rhombomeres, several Eph-related RTKs are expressed in neural crest cells migrating from the hindbrain to the branchial arches, for example, Sek-1, which is expressed in those that migrate from r5 to the third branchial arch (Nieto *et al.*, 1992; Irving *et al.*, 1996). Furthermore, dominant negative interference with Sek-1 function results in disruption to the targetted migration of branchial neural crest (A. Smith *et al.*, in preparation). Our finding that AL-1/RAGS and LERK4 are expressed in the surface ectoderm of the branchial arches raises the possibility that these ligands signal to Eph-related receptors expressed in branchial neural crest cells, which in the mouse migrate underneath the ectoderm (Serbedzija *et al.*, 1992). Since these ligands appear to be expressed in all branchial arches, it seems likely that they have a role in guidance rather than repulsion.

Potential Roles in Neuronal Pathfinding

We find that ELF-1, Elk-L, and LERK4 are expressed in specific glial or neuronal populations, or their precursors, in the spinal cord. Elk-L is expressed in two glial populations: the roof plate, and, after 13 days of development, also in the floor plate. One possibility is that, by analogy with the role of AL-1/RAGS in repulsion, the roof plate expression is associated with the absence of axons crossing the dorsal midline of the spinal cord (Snow *et al.*, 1990). In the case of the floor plate, Elk-L3 is expressed at early stages (Gale *et al.*, 1996b), and thus it is possible that the later up-regulation of Elk-L reflects a similar but stage-specific function of these transmembrane ligands. Since the floor plate has a major role in the organisation of axon tracts, with commisural axons crossing (reviewed by Dodd and Jessell, 1988) whereas those of the primitive longitudinal tract do not (Yaginuma *et al.*, 1990), this raises the question as to whether these ligands might act as attractive or repulsive cues for these axons. It will therefore be interesting to analyse the expression in the spinal cord of receptors for these ligands.

We find that AL-1/RAGS is expressed in a subset of motor neurons, whereas initial data had suggested that ligands are only expressed in the pathway or targets of neurons, which express the corresponding receptor(s). Since the Sek-1 and Mek-4 receptors are expressed in specific motor neurons (Nieto *et al.*, 1992; Ohta *et al.*, 1996; Kilpatrick *et al.*, 1996), it is possible that AL-1/RAGS interacts with these receptors to mediate a repulsion preventing the fasciculation of axons

with distinct targets. Alternatively, or in addition, such interactions could underlie the segregation of cell bodies of different subclasses of motor neurons in the ventral horn. These possibilities predict that there is a complementary expression of receptors and ligands in different motor neurons. Recent data has shown that a null mutation in Nuk receptor leads to defects in the posterior part of the anterior commisural tract, yet these axons express Elk-L, whereas Nuk is expressed in cells along their pathway (Henkemeyer *et al.*, 1996). Since this axon tract forms normally when the kinase domain of Nuk is disrupted and the extracellular domain left intact, this suggests that transmembrane ligands may transduce signals upon binding to receptor.

Potential Roles of Ligands during Limb Development

Previous studies have suggested roles of Eph-related RTKs in the limb based on the expression of Eck (Ganju *et al.*, 1994) and Sek-1 (Patel *et al.*, 1996) in distal mesenchyme during outgrowth, and subsequently in specific differentiating cell types, including perichondrial cells and tendons. Moreover, the distal expression of Sek-1 occurs in a posterior–anterior gradient and has been shown to be regulated by signals from the apical ectodermal ridge (AER) and zone of polarising activity (ZPA) that are implicated in patterning along the limb axes (Patel *et al.*, 1996). Our finding that several ligands are expressed in the developing limb further supports the possibility of roles in patterning of this tissue. The highly dynamic early expression patterns of B61 and Elk-L are particularly intriguing, as these occur in spatial domains that do not correlate in a simple manner with those of previously described genes. B61 expression initially occurs in a circular domain in posterior distal mesenchyme overlapping with Eck and Sek-1 expression, raising the possibility that it mediates a contact-dependant signal in the posterior limb. However, the functional significance of the subsequent elongation of the circle of B61 expression towards the centre of the limb is obscure. Elk-L displays a complicated and dynamic early expression, that includes the distal mesenchyme, and stripes along both the anterior–posterior and proximal–distal axes. One possibility is that the latter relates to the future location along these axes of differentiating components of the limb, and indeed at later stages, several ligands are expressed in patterns that correlate with differentiation of the limb. Elk-L expression appears to be associated with forming tendons and nail beds, whereas ELF-1, LERK4, and AL-1/RAGS are expressed in overlapping but distinct patterns around the forming cartilages, complementary to the expression of Sek-1 and Eck.

These observations raise the question as to whether Eph-related receptors and ligands might have roles in stimulating or restricting cell movement in the developing limb. Previous work has emphasised the potential role of adhesion molecules in limb patterning, and it is interesting to consider whether, as discussed above for other tissues, migration or repulsion might also be involved. For example, the complementary expression of several GPI-anchored ligands and their receptors is consistent with a role in a repulsion that segregates the condensing cartilages from surrounding mesenchyme. Similarly, cell mixing experiments have shown that cells from different regions along the anterior–posterior and proximal–distal axes of the limb bud will sort out from each other (Ide *et al.*, 1994), and we speculate that this may involve repulsion mediated by Eph-related receptors and ligands that normally partially constrains the intermingling of different regions.

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